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Photo(geno)toxicity Changes Associated with Hydroxylation of the Aromatic Chromophores during Diclofenac Metabolism

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Abbreviations: DCF, diclofenac; 5OH-DCF, 5-hydroxy-diclofenac; 4'OH-DCF, 4'-hydroxy-diclofenac; CPZ, chlorpromazine; SDS, sodium dodecyl sulphate; NSAID, nonsteroidal antiinflammatory drug; CYPs, cytochrome P450 enzymes; ssb, single strand break; Fpg, *E. coli* formamidopyrimidine DNA glycosylase; Endo III, *E. coli* endonuclease III; EndoV, T4 endonuclease V; FBS, fetal bovine serum; PBS, phosphate buffered saline; FSK, fibroblasts; DMEM, Dulbecco's modified eagle medium; TAE, tris-acetate-EDTA; NRU, neutral red uptake; PIF, photo-irritation factor.

HIGHLIGHTS

- Hydroxylation of DCF at the aromatic rings modulates its photo(geno)toxic potential
- 5OH-DCF metabolite is phototoxic to cells, as demonstrated by the 3T3 NRU assay
- DNA ssb photosentized by DCF and 5OH-DCF is observed on agarose gel electrophoresis
- Comet assay reveals the photodamage induced by DCF and 5OH-DCF to cellular DNA
- Most DNA photodamage by DCF and 5OH-DCF is repaired by cells after several hours

ABSTRACT

Diclofenac (DCF) can cause adverse reactions such as gastrointestinal, renal and cardiovascular disorders; therefore, topical administration may be an attractive alternative to the management of local pain in order to avoid these side effects. However, previous studies have shown that DCF, in combination with sunlight, displays capability to induce photosensitivity disorders. In humans, DCF is biotransformed into hydroxylated metabolites at positions 4' and 5 (4'OH-DCF and 5OH-DCF), and this chemical change produces non negligible alterations of the drug chromophore, resulting in a significant modification of its light-absorbing properties. In this context, 5OH-DCF exhibited higher photo(geno)toxic potential than the parent drug, as shown by several in vitro assays (3T3 NRU phototoxicity, DNA ssb gel electrophoresis and COMET), whereas 4'OH-DCF did not display significant photo(geno)toxicity. This could be associated, at least partially, with the more efficient UV-light absorption by 5OH-DCF metabolite. Interestingly, most of the cellular DNA damage photosensitized by DCF and 5OH-DCF was repaired by the cells after several hours, although this effect was not complete in the case of 5OH-DCF.

Keywords: Comet assay, DNA repair capability, Metabolites, Photosensitized DNA damage, Phototoxicity

1. INTRODUCTION

Diclofenac (2-(2,6-dichlorophenylamino)phenylacetic acid, DCF) is a widely prescribed nonsteroidal antiinflammatory drug (NSAID), which can be administered either topically or orally. It is therapeutically used in the treatment of several rheumatic diseases and as an analgesic. As DCF can cause severe adverse reactions such as gastrointestinal, renal and cardiovascular disorders, topical administration may be an attractive alternative to the management of local pain in order to avoid these side effects.[1] However, previous studies have shown that DCF, in combination with sunlight, displays capability to induce photosensitivity reactions.[2,3,4,5,6]

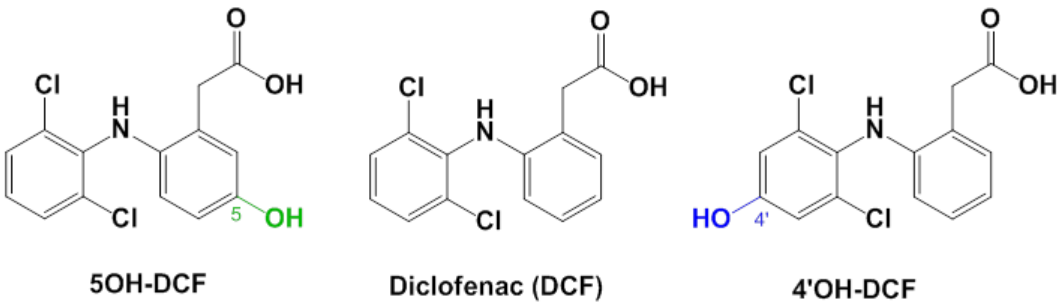
In this context, taking into account that metabolites can generate, upon irradiation, reactive intermediates capable of binding to key biomacromolecules such as DNA, identification of metabolites with phototoxic or adduct forming capability still remains a major challenge. Therefore, although biotransformation is normally associated with a decreased toxicity, metabolites may be more phototoxic and photoreactive than the parent drug.[7,8,9]

Thus, in a previous work we have proven that demethylation of chlorpromazine (CPZ) as a consequence of Phase I biotransformation, does not result in a detoxification but leads to metabolites maintaining identical chromophore to the parent drug and exhibiting an even enhanced phototoxicity.[10]

In humans, DCF is biotransformed into hydroxylated metabolites *via* oxidation of the aromatic rings by cytochrome P450 enzymes (CYPs).[11,12] Major metabolic pathways are the hydroxylation in position 4' and 5 (4'OH-DCF and 5OH-DCF, see Figure 1A), which unlike the case of CPZ demethylation are associated with a change in the chromophore. Moreover, as shown in Figure 1B both metabolites 4'OH-DCF and 5OH-

DCF display a bathochromic shift of the absorption band towards the UVA region, thus extending the active fraction of solar light able to produce photosensitivity disorders. With this background, the goal of the present work is to assess the photo(geno)toxic potential of DCF metabolites in order to investigate whether DCF biotransformation modulates the potential to photosensitize DNA damage.

A)



B)

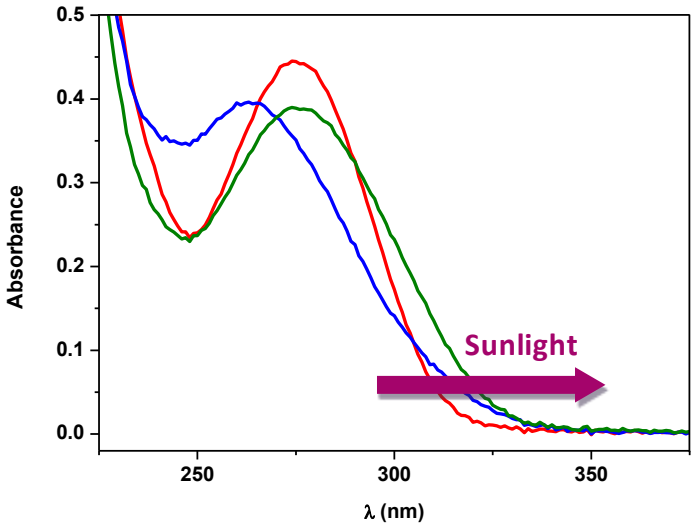


Figure 1. A) Chemical structures of diclofenac (DCF) and its metabolites 4'OH-DCF and 5OH-DCF. B) Ultraviolet spectra of DCF (red), 4'OH-DCF (blue) and 5OH-DCF (green). Concentrations of DCF and its metabolites were 4×10^{-5} M in PBS.

2. MATERIALS AND METHODS

2.1 General chemicals and reagents

All solvents were commercially available (HPLC grade) and were used without any further purification. Diclofenac sodium salt (**DCF**), 4'-hydroxydiclofenac (**4'OH-DCF**), 5-hydroxydiclofenac (**5OH-DCF**) chlorpromazine (CPZ), sodium dodecyl sulphate (SDS), neutral red solution and DNA repair enzymes *E coli* formamidopyrimidine DNA glycosylase (Fpg) and *E coli* endonuclease III (Endo III) were provided by Sigma Aldrich (Madrid, Spain). For the preliminary experiments, 5-hydroxydiclofenac (**5OH-DCF**) was synthesized by standard procedures.[13] Supercoiled plasmid pBR322, DNA repair enzyme T4 endonuclease V (EndoV) and SYBR Safe DNA gel stain were purchased from Roche Diagnostics (Barcelona, Spain), Ecogen (Barcelona, Spain) and Invitrogen (Madrid, Spain), respectively. For cell culture experiments, fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM) and penicillin-streptomycin were supplied by Invitrogen (Madrid, Spain) and trypsine-EDTA (0.25%-0.02%) and glutamine (100 mM) solutions were provided by Cultek (Madrid, Spain). Phosphate buffered saline solution (PBS, pH 7.4, 0.01 M) was prepared by dissolving Sigma tablets in the appropriate volume of ultrapure deionized water. Reagent kit for single cell electrophoresis assay was supplied by Trevigen (Barcelona, Spain).

2.3 UV Absorption spectra

Ultraviolet absorption spectra were recorded on a Shimadzu UV-1800 UV/VIS spectrophotometer. Measurements were performed in PBS (4×10^{-5} M) at room temperature using 1 cm quartz cells with 3.5 mL capacity.

2.4 Irradiation equipment

The UV light source used in all *in vitro* photosensitization assays was a photoreactor model LZC-4 (Luzchem, Canada) equipped with 14 lamps for top and side irradiation (λ_{max} = 350 nm, Gaussian distribution). All irradiations were carried out through the lid of the plates, and in order to avoid overheating ventilation was used and the plates were placed on ice during the irradiation step.

2.5 DNA damage induced by photosensitization

Mixtures containing 200 ng of supercoiled circular plasmid pBR322 and DCF (100 μM) or its metabolites (100 μM) were irradiated as described above. Digestion with an excess of the repair enzymes (Fpg, Endo III or Endo V) was performed immediately after UVA irradiation in order to reveal the nature of DNA damages. Upon irradiation/digestion, loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol, 30 % glycerol in water) was added to each sample. All the samples were loaded on a 0.8 % agarose gel containing SYBR® Safe as dye of nucleic acid. Electrophoresis was carried out in Tris-acetate-EDTA (TAE) buffer (0.004 M Tris-acetate, 1 mM EDTA) at 100 V for 1 h. Next, the DNA bands were detected under UV light irradiation and visualized using a Gel Logic 200 Imaging System (Kodak). Finally, the relative abundance of supercoiled DNA (Form I) and nicked relaxed DNA (Form II) was quantified by densitometry with the image analyzer Quantity One (Biorad).

2.6 Assessment of cellular photo(geno)toxicity

2.6.1 In Vitro 3T3 neutral red uptake (NRU) phototoxicity test

BALB/c 3T3 fibroblast cell line was grown in DMEM supplemented with 10% FBS, 4 mM glutamine and 1% penicillin/streptomycin and routinely maintained in 75 cm^2

plastic flasks in a humidified incubator at 37 °C under 5 % CO_2 atmosphere. The 3T3 NRU phototoxicity test was performed according to the OECD guideline 432[14] with minor modifications. For each compound two 96-wells plates were seeded at a density of 2.5×10^4 cells/well. Serial dilutions of the test compounds ranging from 4 mM to 0.05 mM were added to each plate. After a period of 1h incubation, one plate was irradiated with a dose of UVA equivalent to 5 J/cm^2 (UVA light), whereas the other plate was kept in a dark box (dark). The viability of UVA-treated control cells in the absence of test compounds was >90% of those kept in the dark indicating the suitability of the UV dose. At the end of the UVA exposure plates were replaced with DMEM medium and then incubated overnight. Next day neutral red solution (50 μg/mL) was added into each well and incubated for 2 h. After that, neutral red medium was discarded, cells were washed with PBS and neutral red extraction was achieved with 100 μL of the desorption solution (water 49% (v/v), ethanol 50% (v/v) and acetic acid 1% (v/v). The absorbance was recorded at 550 nm on a Multiskan Ex microplate reader. For each compound dose-response curves were developed, which allowed the determination of IC_{50} values (concentration of compound causing a 50% reduction of the neutral red uptake) in the absence and in the presence of radiation. Finally, the Photo-Irritation-Factor (PIF) was calculated with the following equation: $PIF = \frac{\text{IC50 DARK}}{\text{IC50 UVA LIGHT}}$. According to the OECD Guideline[14] a test compound is labeled as "phototoxic" if PIF is >5, "probably phototoxic" if PIF >2 and <5, and "non-phototoxic" when PIF <2. CPZ and SDS were used as positive and negative controls, respectively.

2.6.2 Nuclear DNA damage by COMET assay

The single cell gel electrophoresis assay, also known as comet assay, was performed as previously described[10] with slight modifications. Human fibroblasts (FSK cell line) were trypsinized, resuspended in cold PBS and placed on ice for 2h. Cells (100000 cells/well in two 12-well plates) were seeded and treated with 100 μM of DCF or its metabolites. CPZ (10 μM) was used as a positive control. After 1h incubation, one plate was placed in the photoreactor in order to irradiate the cells for 5 min on ice, whereas the other one was kept in a dark box. Next, 100 μL of each cell suspension were mixed carefully with 100 μL of 1% low melting point agarose solution and drops were loaded onto Trevigen® treated slides and placed on ice-cold tray to allow its jellification. Then the slides were immersed in coupling jars containing cold lysis buffer (2.5M NaCl, 0.1 M Na_2EDTA, 0.01 M Tris, 1% Triton X-100 in distilled water and pH 10) and overnight incubated at 4 °C. In DNA-recovery assays, after drop jellification, the slides were incubated in DMEM medium at 37 ºC for different time periods (3h, 6h or 18h) and then subjected to cell lysis. Next day all slides were placed in a Trevigen® Comet assay electrophoresis tank (10 slides per run), covered with 850 mL of cold alkaline electrophoresis buffer (0.2 M NaOH, 1mM EDTA in distilled water and pH ≥13) and let during 40 min for DNA unwinding at 4 °C. Afterwards, the electrophoresis was run at 21 V (≈300 mA) for 30 min at 4 °C. When the electrophoresis finished, the slides were neutralized twice in PBS for 5 min and washed once with miliQ water for 5min; DNA was fixed by slide incubation in 70% ethanol for 5 min followed by other 5 min in 100% ethanol, and then air-dried. Finally, comet nucleoids and tails were stained by incubating the slides in a SYBR Gold® (1:10000 TE buffer) bath for 30 min, air dried, and kept in darkness until further visualization. For nucleoids and tails DNA visualization a Leica DMI 4000B fluorescence microscope was used and ≥5 pictures

were taken for each sample. Finally, DNA % in tail as a measure of DNA damage was determined for each condition with the analysis of at least 100 DNA comets with the open source analysis software Open Comet 1.3[15]

3. RESULTS AND DISCUSSION

3.1 Phototoxicity

In vitro 3T3 NRU phototoxicity assay was performed in order to assess the cell viability upon exposure to DCF and its metabolites in combination with UVA irradiation (5 J/cm^2). Accordingly, cytotoxicity profiles of BALB/c 3T3 fibroblasts treated with DCF, 4'OH-DCF and 5OH-DCF were measured, using neutral red as vital dye, both in dark and in the presence of UVA light. Thus, the IC_{50} values were determined from dose-response curves for cell viability of cells treated under the conditions described above in the materials and methods section (dose-response curves are provided in Supplementary material, Figure S1).

The aim of NRU test is to calculate the PIF that corresponds to the ratio of the IC_{50} under dark or light conditions for each compound. As shown in Table 1, 5OH-DCF metabolite resulted to be potentially phototoxic with a PIF value *ca.* 12, 4 fold more phototoxic than the parent drug. Both DCF and 4'OH-DCF displayed a PIF of 3 and 2, respectively, which can be considered as probable phototoxicity. Table 1 collects the IC_{50} under dark and UVA light conditions as well as the PIF of all compounds tested.

It is known that the photosensitizing properties of DCF are associated with the main photoproduct, which corresponds to a chlorocarbazole derivative, as revealed in photohemolysis and lipid photoperoxidation assays.2 Thus, the key process is the photodechlorination, triggered by homolytic carbon-chlorine bond cleavage with generation of a highly reactive aryl radical and choride anion.[16] Assuming that this reaction occurs in the parent drug as well as in the metabolites under study, the higher

224 phototoxicity of 5OH-DCF could be associated, at least partially, with its more efficient
225 UV-light absorption.

226 **Table 1.** Phototoxicity of DCF and its metabolites in the 3T3 NRU Assay

Compound	IC_{50} Dark (µM)	IC_{50} UVA Light (µM)	Photoirritant Factor (PIF)[1]
CPZ	84 ± 18	4 ± 1	**21**
DCF	688 ± 63	254 ± 58	**3**
4'OH-DCF	766 ± 85	375 ± 55	**2**
5OH-DCF	813 ± 106	68 ± 27	**12**
SDS	202 ± 25	244 ± 48	**1**

227
228 Data are the mean± SD of five independent experiments performed in triplicate. CPZ
229 and SDS were used as positive and negative controls of phototoxicity respectively.

230 [1]According to the OECD 432 Guide (2004), PIF<2 predicts "no phototoxicity",
231 2<PIF<5 predicts "probable phototoxicity" and PIF>5 predicts "phototoxicity".

232
233 **3.2 Photogenotoxicity of DCF and its metabolites**

234 Irradiations of DCF, 4'OH-DCF and 5OH-DCF in the presence of supercoiled circular
235 DNA (pBR322) were performed using a multilamp photoreactor (λ_{max} = 355 nm) in
236 order to detect DNA damage. Quantification by densitometry of the conversion of
237 native supercoiled form I into circular form II (Figure 2A) shown in agarose gel (Figure
238 2B) evidenced single strand break (ssb) formation. It is interesting to note that 5OH-
239 DCF exhibited higher photogenotoxic potential than the parent drug whereas 4'OH-
240 DCF did not display significant photogenotoxicity (Figure 2).

241 Moreover, in order to reveal the nature of damages induced on the DNA bases, different
242 DNA–repair enzymes were used: i) T4 endonuclease V (Endo V) for cyclobutane

thymine dimers (CPDs), ii) endonuclease III (Endo III) for degradation products of pyrimidine bases and iii) formamidopyrimidine DNA glycosylase (FPG) for oxidized purines. Thus, ssb formation was enhanced for 5OH-DCF metabolite (data reported in Supplementary material, Figure S2) in all cases.

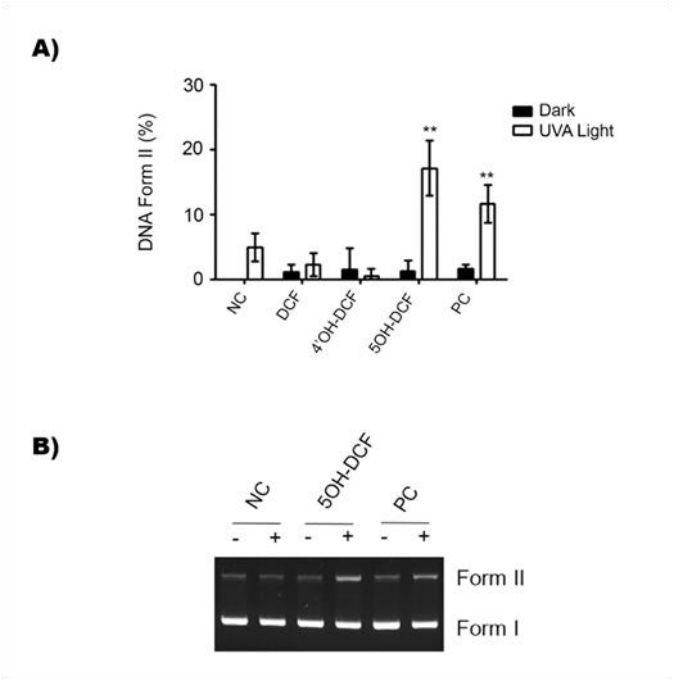


Figure 2. A) Induction of single strand brakes (ssb) in supercoiled circular pBR322 plasmid (200 ng/µL) alone (negative control, NC) or treated with DCF and its metabolites at 100 µM upon 30 minutes UVA irradiation (□) or not (■) using a multilamp photoreactor λ_{max} = 350 nm. DNA Form II was quantified by densitometry of agarose gel electrophoresis. Data represent the mean ± SD of four independent experiments and asterisks indicate significant differences relative to the formation of DNA Form II in Dark conditions by the T-Student test (**$p<0.01$). B) Agarose gel electrophoresis of plasmid pBR322 alone (NC) or in the presence of 5OH-DCF, in the dark (-) or upon 5 minutes UVA irradiation (+). Ketoprofen was used as positive control (PC).

Assessment of cellular photogenotoxicity was performed by Comet assay under alkaline conditions to reveal the combined nuclear DNA damage resulting from single-strand breaks, double-strand breaks and alkali-labile sites. For this purpose, human fibroblasts (FSK) were embedded in agarose on a slide and incubated for 1 h with DCF and its hydroxylated metabolites. Next, alkaline electrophoresis was carried out after 5 min of UVA exposure (2 J/cm^2) and subsequent lysis. In the course of electrophoresis, the damaged and fragmented DNA migrates away from the nucleus and upon staining with SYBR Gold the fluorescence of the nuclei was observed. Percentage of DNA damage was calculated by means of OpenComet software.

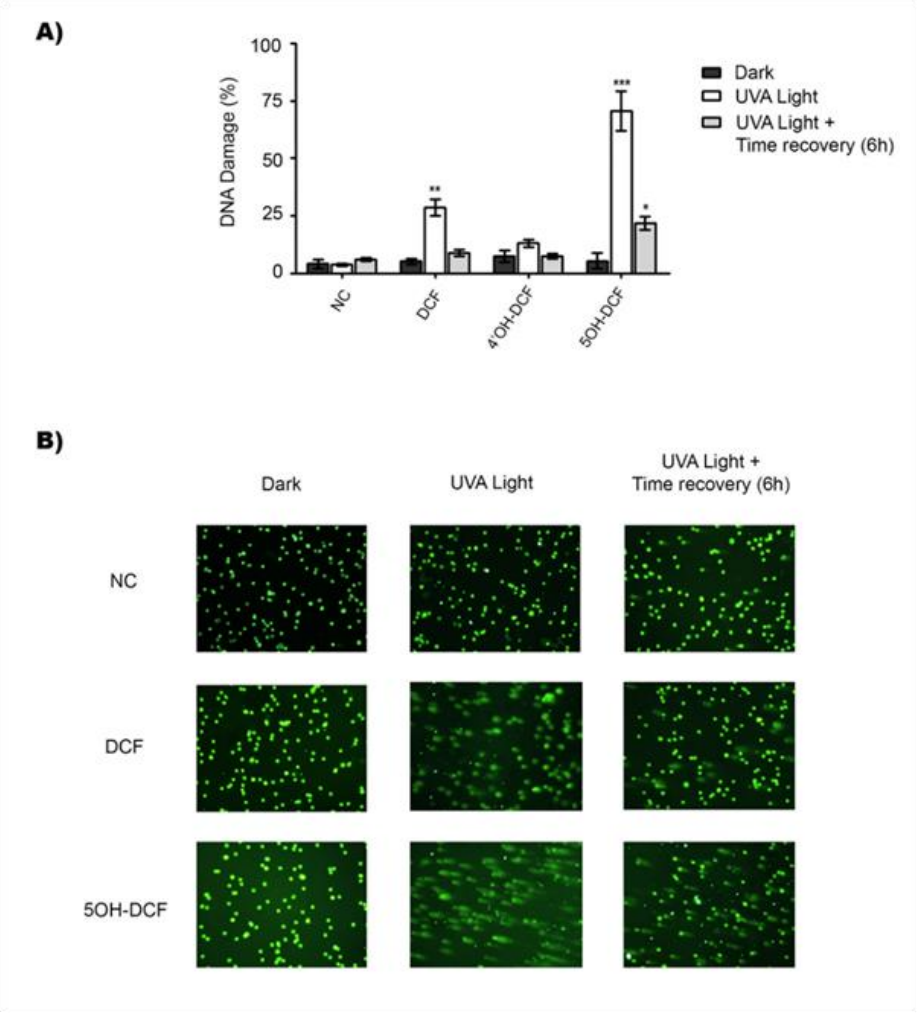


Figure 3. Alkaline comet assay and DNA repair capability of FSK cells treated with DCF and its metabolites. A) Percentage of DNA damage calculated by OpenComet software of untreated FSK cells (Negative control, NC) or treated with DCF and its metabolites (100 μM). Cells were left unexposed (Dark, ■), irradiated for 5 minutes (UVA Light, □) or irradiated for 5 minutes followed by 6 h of cell recovery (UVA Light + Time recovery 6h, ■), respectively. Data are the mean ± SD of three independent experiments. Asterisks indicate significant differences relative to the untreated FSK cells in dark conditions by the T-Student test (*$p<0.05$; **$p<0.01$; ***$p<0.001$). B) Fluorescence microscopy images of DCF and 5OH-DCF Comet assay experiments.

As shown in Figure 3, 5OH-DCF showed again higher photogenotoxicity than the parent drug DCF and 4'OH-DCF metabolite (see Supplementary material, Figure S3). Moreover, another set of experiments were performed in order to investigate the ability of FSK cells to repair nuclear DNA damage generated by DCF and metabolites in combination with UVA light. For this purpose, FSK cells treated (with drug and metabolites) and irradiated were incubated for different time periods (3h, 6h and 18h) and the remaining DNA damage was calculated as described above. As a general trend DNA damage decreased with the time of recovery. Interestingly, for DCF it was shown that most DNA damage was repaired within 3h after irradiation whereas for 5OH-DCF a significant residual DNA damage (around 25%) was still present even after 18 h of cell recovery (Figure 3 and Supplementary material, Figure S4).

4. CONCLUSION

Hydroxylation of the aromatic rings at positions 4' and 5 occurs during phase I biotransformation of DCF. This chemical change produces non negligible alterations of the drug chromophore and results in a significant modification of its light-absorbing

properties. Accordingly, the phototoxic and photogenotoxic potential of DCF, 4'OH-DCF and 5OH-DCF are expectedly different, as indicated by 3T3 NRU phototoxicity assay, the DNA single strand break gel electrophoresis assay and the COMET assay. The most remarkable result is the enhanced photo(geno)toxicity of 5OH-DCF, which was consistently observed in all experiments. Interestingly, most of the cellular DNA damage photosensitized by DCF and 5OH-DCF was repaired by the cells after several hours, although this effect was not complete in the case of 5OH-DCF.

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